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# Influence of resting tension on protease-activated receptor-mediated relaxation in guinea-pig tracheas

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**Abstract**

We investigate the role of resting tension on thrombin (THR) induced relaxation of guinea-pig tracheas precontracted with acetylcholine (ACh).

Isometric contractions of isolated guinea-pig tracheas were recorded at 4 and 6 g resting tension; and ACh dose–response curves were performed.

THR relaxed ACh-precontracted tracheas and this effect was mimicked by the type 2 protease activating receptor agonist peptide (PAR-2 AP) and trypsin.

The relaxant effect of 3 U $ml^{-1}$ THR and 100 nmol $ml^{-1}$ PAR-2 AP was prevented at 4 g by preincubation with the nitric oxide synthase (NOS) inhibitor L-NAME and at 6 g resting tension by ibuprofen and diclofenac. However, adenosine trisphospahate (ATP) relaxation was totally prevented by cyclooxygenase (COX) inhibitors but not by NOS inhibitors at both resting tensions.

Resting tension influenced the effect of $PGE_2$ on contractile tone of isolated guinea-pig tracheas, the maximal relaxation being $-11.1 \pm 2.97$ and $-2.0 \pm 0.46$ mg $mg^{-1}$ tissue wet weight at 6 and 4 g, respectively. Moreover, 30 nmol $ml^{-1}$ $PGE_2$ can relax ACh-precontracted tracheas, being the effect up to 91 and 30% at 6 and 4 g, respectively.

These data demonstrate that trachea responsiveness is highly dependent on the smooth muscle length, revealing new aspects of stretch-activated receptors that can influence trachea responsiveness in vivo.


*Keywords:* Thrombin; Trypsin; Type 2 protease activating receptor agonist peptide; Nitric oxide; Prostaglandin E2

## 1. Introduction

In recent years, protease activated receptors (PARs) have been described in several tissues, including the airways (for a short review see [1]). In particular, the cytoprotective activity of thrombin, trypsin and tethered-ligand peptides SLIGRL-NH2 for PAR-2 and SFLLIRN-NH2 for PAR-1 has been originally described in mouse, rat, guinea-pig and human isolated bronchi with epithelium [2]. In vitro, both proteases can relax ACh-precontracted bronchi, this relaxant effect being mainly mediated by the activation of PAR-2 localised in the epithelium. In the absence of epithelium, both peptides are ineffective. Moreover, in vivo aerosol administration of SLIGRL-NH2 inhibits serotonin-induced bronchoconstriction in rats [2]. The relaxing effect in vitro of proteases is prevented by the cyclooxygenase inhibitors indomethacin and acetylsalicylic acid, suggesting an involvement of cyclooxygenase metabolites in the PAR-mediated relaxant effect. On the other hand, the block of NO production does not influence the relaxing effect of both peptides [2]. Subsequent research confirms the complex role of proteases and their receptors in airway function, demonstrating that PARs are localized not only in

*Abbreviations:* ACh, acetylcholine; ATP, adenosine 5′-triphosphate; COX, cyclooxygenase; L-NAME, ω-nitro-L-arginine methylester; NOS, nitric oxide synthase; $PGE_2$, prostaglandin $E_2$; PARs, protease-activated receptors; PTIO, 2-phenyl-4,4,5-tetramethyl-imidazoline-1-oxyl-3-oxide; THR, thrombin.

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the bronchial tree, but also in the trachea and peripheral lungs [1].

The isolated guinea-pig trachea also reacts to proteases. According to the different in vitro preparations and experimental conditions used, proteases can mediate either relaxation [3] or contraction [4]. Moreover, the intravenous administration of the PAR-2 activating peptide (PAR-2 AP) SLIGRL (1 mg $kg^{-1}$) can prevent in vivo histamine-induced bronchoconstriction in guinea-pigs [5]. Also the involvement of cyclooxygenase metabolites and NO in the relaxing effect of PARs agonists has been further investigated in guinea-pigs [3,5]. Whereas the inhibition of COX by indomethacin, and the inhibition of nitric oxide synthetases (NOS) by ω-*N*-monomethyl-L-arginine (L-NMMA) can decrease the relaxant effect of PAR-2 activating peptide SLIGRL-NH2 in guinea-pig tracheas in vitro [3], indomethacin and ω-nitro-L-arginine-methylester (L-NAME) in vivo are unable to counteract the relaxant effect of PAR-2 AP [5]. According to Carr et al. [4], trypsin can induce contractions in indomethacin-pretreated tracheal preparations, this effect being prevented by neurokinin receptor antagonists (type1 receptor, NK1). Therefore, several discrepancies on PARs actions are still unexplained in literature.

In the mouse trachea, prostaglandin $E_2$ ($PGE_2$) is mainly involved in this relaxant effect of PARs agonist, acting through its E-prostanoid type 2 (EP2) receptor as demonstrated with the DP/$EP_1$/$EP_2$ prostanoid receptor antagonist AH6809 [6]. When comparing $PGE_2$ effects on wild-type and EP receptor-deficient mice, the tracheal relaxation due to this lipid autacoid is dependent on EP2 receptors [7,8]. Moreover, ATP and substance P increase $PGE_2$ release that in turn stimulates EP2 receptors, thus mediating the relaxant effect of $PGE_2$ as determined with an EP2-deficient mice model [7].

According to Asokananthan and co-authors [9], PAR-1, PAR-2 and PAR-4 receptor agonists can stimulate the release of $PGE_2$ in human pulmonary epithelial cell lines (A549 and HBECs). All peptides are similarly effective on the A549 cell line, while the HBEC cell line produces a 10-fold larger amount of $PGE_2$ both at baseline and after stimulation and is more responsive to the PAR-2 receptor agonist.

Old data obtained in guinea-pig tracheal strips [10], demonstrate that $PGE_2$ as well as ATP are contractile agonists at low resting tone, but relaxing agents when the resting tension is set at high values and when the tension is increased by histamine pre-treatment. Similar results are also described by Advenier and co-authors [11] regarding the effect of ATP and adenosine on guinea-pig tracheas. Both molecules can relax ACh precontracted preparations, but contract guinea-pig tracheas when the only tension applied is resting tension. More recently, Watson et al. [12] demonstrate that resting tension can influence the contractile response of isolated human bronchial ring preparations. Although the contraction induced by 8 Hz electrical field stimulation is monophasic independently of the applied resting tension, when electrical field stimulation frequency is increased up to 30 Hz, the contractile phase is followed by a relaxation when preparations are stretched at high resting tension. However, this biphasic behaviour is absent when resting tension is set at lower values than the initial one. In the same study, resting tension dependency is also established by testing the effect of isoprenaline and carbachol. Isoprenaline induces a significantly improved relaxation when resting tension is set at low values [12]. Moreover, the contractile potency of carbachol (3 μM) is only marginally decreased when the resting tension is high. On the other hand, at high resting tension and when preparations after carbachol challenge are extensively washed (60 min), bronchial tissue specimens recover to significantly lower tension values than those measured before muscarinic stimulation.

According to preliminary data obtained in our laboratory, the applied isometric stretching (i.e. resting tension) can modify smooth muscle responsiveness to several agonists. Stretch (resting tension) is an essential determinant of muscular fibre length and it can mimic experimentally the change in smooth muscle length related to the physiological breathing process, when the airways are submitted to cyclic changes in volume.

Therefore, we decided to investigate the possible influence of resting tension on PARs responsiveness in isolated guinea-pig trachea since no effort have been made to relate the PARs reactivity to this experimental parameter. In order to better clarify this point, ATP was mainly used to compare PARs response to a more traditional relaxing agent, but since several experiments had revealed peculiar aspects of ATP pharmacology, numerous data on ATP were also described below.

## 2. Methods

This investigation conforms to European Union rules for the care and use of laboratory animals.

Pathogen-free white male guinea-pigs (300–350 g) were obtained from Harlan (Italy), housed under controlled conditions and used at least two weeks after their arrival in our animal facilities. Commercial chow and water were allowed ad libitum. Animals were anaesthetised with sodium thiopental (80 mg $kg^{-1}$ i.p.) and killed by cervical dislocation. Tracheas were dissected free of connective tissue and placed in cold gassed (5% $CO_2$ in $O_2$) Krebs–Henseleit solution (K–H) of the following composition (mM): 110 NaCl, 25 $NaHCO_3$, 4.8 KCl, 1.2 $KH_2PO_4$, 1.2 $MgSO_4$, 11 mM D(+)glucose, 2.5 mM $CaCl_2$ and cut into helical strips (3 mm width, 20 mm length). Strips were mounted in an organ bath (2 ml) with one end attached to a tissue holder and the other to a research grade isometric force transducer (Harvard Apparatus, Inc., South Natick, Massachusetts, USA) connected to an analogical/digital converter. Signal was

elaborated by Window-based software (RCS, Florence, Italy); the real-time tissue response data were continuously collected, displayed on the computer screen and recorded as ASCII data files. Strips were perfused with pre-warmed (37 °C) K–H, gassed with 5% $CO_2$ in $O_2$.The resting tension was set at 4 or 6 g and the tissue was equilibrated for at least 60 min before starting with experimental protocols. These resting tensions were arbitrarily chosen in the range in which ACh induced an appreciable contraction and its dose–response curve was superimposable. After equilibration, tension was assumed as 0 mg $mg^{-1}$ tissue wet weight (w.w.). Cumulative dose–response curves were performed by adding as a bolus incremental doses of ACh to the closed 2 ml organ bath. The maximal contraction obtained was expressed as 100% of contractile response and the cumulative dose–response curve of ATP and thrombin (THR) was made using the maximal ACh dose (100%). The functionality of each preparation was tested in control conditions by assessing the relaxant effect of ATP in ACh-precontracted trachea strips. However, using ATP we obtained distinguishing data, that therefore were reported in the result section. Experiments were mostly performed on epithelium-intact preparations; in several experiments the epithelial layer was rubbed with a cotton gauze and tracheas were then mounted as above indicated. ACh, THR, trypsin, ATP, and PARs peptides were administered as a bolus injection to the closed 2 ml organ bath. Since the effect of proteases implies a proteolytic degradation of their receptors, experiments aimed to test the effect of proteases in different experimental conditions were run in parallel on two different tracheal preparations. According to the experimental protocol, after assessing functionality in control conditions, tracheas were perfused (5 ml $min^{-1}$) with K–H containing L-NAME, 2-phenyl-4,4,5-tetramethyl-imidazoline-1-oxyl-3-oxide (PTIO, a NO trapping agent, [13]), ibuprofen or diclofenac at specified concentrations for at least 30 min (open organ bath) before the ACh bolus administration and maintained throughout the remaining experimental time. Cumulative dose–response curves were also performed by adding incremental doses of $PGE_2$ as a bolus to the closed 2 ml organ bath. In several experiments, PAR-2 was administered to the ACh-precontracted trachea and ATP, THR or trypsin were administered to the already relaxed specimen.

### 2.1. $PGE_2$ quantification

$PGE_2$ tissue release in the organ bath was quantified using radioimmune [$^{125}$I] assay (RIA) kits (Amhersham Biosciences UK Limited, Buckinghamshire England). Briefly, at the end of stabilisation time, the organ bath was closed and its content was carefully collected after 6 min and immediately frozen at −80 °C. The organ bath was then rapidly replenished, 10 nmol $ml^{-1}$ ACh administered and after 3 min a bolus dose of the buffer alone was added and, after an additional 3 min period, the organ bath medium was collected and stored at −80 °C. The organ bath was refilled and 3 min after ACh administration (10 nmol $ml^{-1}$), strips received either 100 nmol $ml^{-1}$ ATP or 3 U $ml^{-1}$ THR, and the organ bath buffer collected after 3 min. Then, the above-described procedure was repeated, switching from ATP to THR and vice versa. Preparations received first ATP or THR alternatively. Preparations were then incubated for 60 min with 1 μM diclofenac (perfusion) and the entire protocol was repeated, maintaining diclofenac throughout the experiment. The collecting time of 3 min + 3 min was selected as necessary and sufficient to obtain the ACh maximal contractile value as well as to obtain the complete relaxation after ATP or THR. Experiments were performed on different tracheas at 4 and 6 g resting tension. The functional real-time response of each preparation was continuously monitored. Collected media were immediately lyophilised and processed for $PGE_2$ dosage as described by the manufacturer. The $PGE_2$ standard was also lyophilised in K–H solution and run similarly. The assay was performed in duplicate. Method sensibility was 8 pg $ml^{-1}$.

### 2.2. Nitric oxide quantification

NO tissue release in the organ bath was quantified using a chemiluminescence NO analyser (model 280 NOA; Sievers; Boulder, CO, USA). The protocol used to collect organ bath medium was very similar to that for quantifying $PGE_2$ release. However, after collecting organ bath media in control conditions, strips were incubated for 30 min with 100 μM L-NAME. Media immediately frozen at −80 °C in sterile test tubes were directly used for NO dosage. The calibration curve was performed by dissolving $NaNO_2$ directly in K–H buffer and processing the standards similarly to the unknown samples. Method sensibility was 1 nmol $l^{-1}$.

### 2.3. Materials

Trypsin, acetylcholine (ACh), ibuprofen sodium salt, diclofenac sodium salt, and 2-phenyl-4,4,5-tetramethyl-imidazoline-1-oxyl-3-oxide (PTIO) were obtained from Sigma Chemical Co. (St Louis, MO, USA); thrombin (from bovine blood) from Roche Molecular Biochemicals (Switzerland) and units, as referred in the text, were measured using the chromogenic substrate Chromozym® TH; ω-nitro-L-arginine methylester (L-NAME) from Calbiochem (La Jolla, CA, USA); *S*-nitroso-*N*-acetylpenicillamine (SNAP) from Tocris Cookson Ltd (North Point, UK), $PGE_2$ from Cayman Chemical (Ann Arbor, MI, USA); PAR-1 (SFLLIRN-NH2) from BACHEM (Bubendorf, Switzerland); PAR-2 (SLIGKV-NH2), PAR-3 (TFRGAP-NH2) and PAR-4 (GYPGKF-NH2) peptides from Inalco (Milan, Italy). The concentration, amino acid composition and purity were checked by the vendors.

### 2.4. Mathematical and statistical methods

Values are presented as means ± s.e. mean. Dose–response curves were analysed with linear regression and compared with one-way ANOVA followed by Bonferroni's $t$ test. Statistical comparisons between data groups were performed using either one-way ANOVA followed by Bonferroni's $t$ test or independently Student's $t$ test as indicated. A $P$ value $\leq 0.05$ was considered significant.

## 3. Results

### 3.1. Effect of ACh

Acetylcholine caused a dose-dependent contraction of tracheal strips at both resting tensions studied (Fig. 1, $r=0.970$, $P<0.01$ and $r=0.976$, $P<0.001$ at 4 and 6 g, respectively); these dose–response curves were superimposable in the full range tested. Moreover, tracheas precontracted with 10 nmol ml$^{-1}$ ACh were still able to increase tensions up to 10-fold (up to 30 mg mg$^{-1}$ tissue wet weight) when a depolarising dose of 100 μmol ml$^{-1}$ KCl was added. In order to investigate the effect of PAR agonists at the different resting tensions we used the maximal contractile bolus dose of 10 nmol ml$^{-1}$ ACh.

### 3.2. Effect of ATP

ATP induced a dose-dependent relaxation of ACh-precontracted tracheal strips (Fig. 2, $r=-0.988$, $P<0.05$ and $r=-0.998$, $P<0.01$ at 4 and 6 g, respectively). These dose–response curves were superimposable, 100 nmol ml$^{-1}$ ATP being able to induce up to 30% of relaxation. However, in epithelium-rubbed preparations, ATP was ineffective at both resting tensions. It should be noted that in the absence of epithelium, 10 nmol ml$^{-1}$ ACh induced a contraction of 4.6 ± 0.27 mg mg$^{-1}$ tissue (w.w., $n=3$) and 5.0 ± 0.19 mg mg$^{-1}$ tissue (w.w., $n=3$) at 4 and 6 g, respectively. These values were significantly different from those obtained in the presence of epithelium ($P<0.05$ and $P<0.001$ at 4 and 6 g, respectively, Student's $t$ test for independent data).

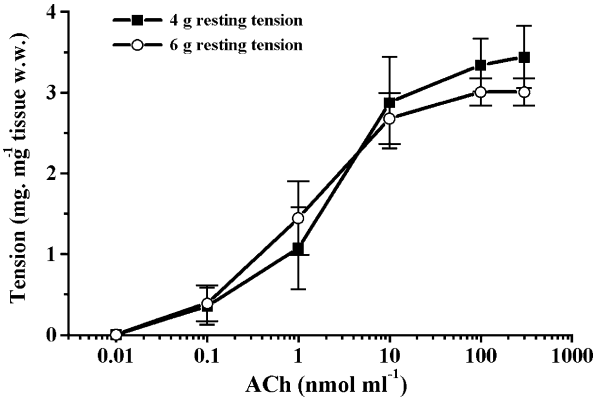


Fig. 1. Effect of acetylcholine (ACh) on isolated guinea-pig tracheal strips. Cumulative dose–response curves of acetylcholine were performed at 4 and 6 g resting tension. ACh was administered as a bolus. Values are the mean ± s.e.m. of at least four experiments.

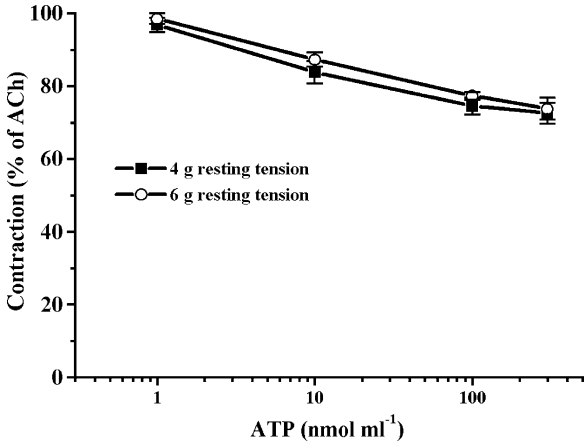


Fig. 2. Effect of adenosine triphosphate (ATP) on 10 nmol ml$^{-1}$ ACh-precontracted guinea-pig tracheal strips. The contraction obtained with ACh was set as 100%. Cumulative dose–response curves of ATP were performed at 4 and 6 g resting tension. ATP was administered as a bolus. Values are the mean ± s.e.m. of at least four experiments. For more details, see Section 3.

Independently of resting tension, the relaxation induced by 100 nmol ml$^{-1}$ ATP was prevented when tracheal strips were preincubated with COX-inhibitors (Fig. 3, panel A). As shown, 10 μM ibuprofen and 1 μM diclofenac were similarly effective in inhibiting the relaxation induced by ATP. On the other hand, COX-inhibitors did not significantly modify ACh-induced contraction (not shown).

Then we tested if endogenous NO contributes to the relaxation induced by ATP. Strips were therefore pretreated with the NOS inhibitor L-NAME (100 μM in perfusion for 30 min); however, as shown in Fig. 3 (panel B), the relaxant effect of 100 nmol ml$^{-1}$ ATP was unaffected. 1 μM PTIO behaved similarly to L-NAME (not shown). Indeed, neither 100 μM L-NAME nor 1 μM PTIO were able to modify either ACh-induced tracheal contraction or ATP-induced relaxation.

### 3.3. Effect of PARs agonists

THR can dose-dependently (0.3–10 U ml$^{-1}$) relax ACh-precontracted tracheal strips, with nearly 50% relaxation being induced by a dose of 3 U ml$^{-1}$. The superimposable dose–dependent curves of thrombin are shown in Fig. 4 ($r=-0.991$, $P<0.01$ and $r=-0.983$, $P<0.05$ at 4 and 6 g, respectively). Similarly to ATP, THR was also ineffective in relaxing epithelium-rubbed preparations at both resting tensions. However, in 2 out of 4 preparations a small contractile increase (6%) was observed when resting tension was set at 4 g and in 3 out of 4 preparations stretched at 6 g.

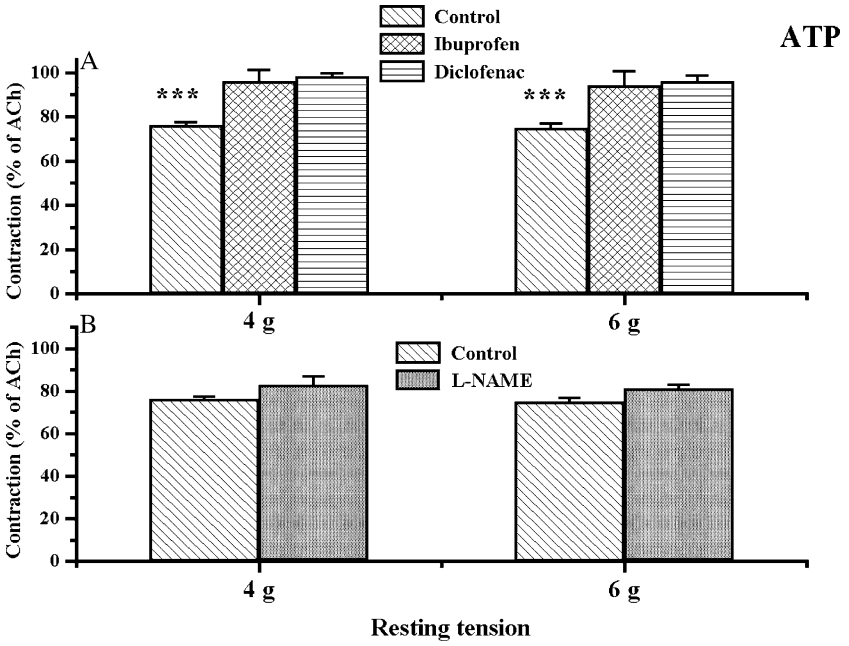


Fig. 3. Effect of COX inhibitors (panel A) and a NOS inhibitor (panel B) on ATP-induced relaxation of 10 nmol $ml^{-1}$ ACh-precontracted guinea-pig tracheal strips stretched at 4 and 6 g as indicated. ATP (100 nmol $ml^{-1}$) was administered in control conditions or after 30 min preincubation with 100 μM ibuprofen, 1 μM diclofenac (panel A) or 100 μM L-NAME (panel B). The contraction obtained with ACh was set as 100%. Values are the mean ± s.e.m. of at least four experiments. For more details, see Section 2. Panel A: ***$P<0.001$ vs. all other treatments, one-way ANOVA followed by Bonferroni's $t$ test.

We then investigated the role of COX-metabolites and NO in THR-induced relaxation using 3 U $ml^{-1}$ THR. As shown in Fig. 5 (panel A), THR-induced relaxation was not modified by COX-inhibitors when tracheas were stretched at 4 g. However, when resting tension was set at 6 g, preincubation with either 10 μM ibuprofen or 1 μM diclofenac totally prevented THR-induced relaxation (Fig. 5(A)).

When tracheal strips were stretched at 4 g, the relaxation induced by thrombin was significantly prevented by the preincubation with 100 μM L-NAME (Fig. 5(B)). At the same resting tension, the preincubation with 1 μM PTIO also reduced the relaxing effect of thrombin (65.3 ± 0.88%, $n=4$ vs. 55.1 ± 5.89% control obtained in parallel experiments, $P<0.05$ independent Student's $t$ test). On the contrary, when resting tension was set at 6 g, thrombin relaxation was not influenced by L-NAME (Fig. 5(B)) or PTIO (not shown).

We also performed experiments using PARs activating peptides (PARs AP). Of the peptides tested, only the PAR-2 AP was effective in relaxing ACh-precontracted tracheas, its effectiveness being very similar at both resting tensions. On the contrary, PAR-1 AP, PAR-3 AP and PAR-4 AP were ineffective up to 100 nmol $ml^{-1}$. The relaxant effect of PAR-2 AP was dose-dependent in the range 1–300 nmol $ml^{-1}$ ($r=-0{,}980$, $P<0.05$). All subsequent experiments were performed using 100 nmol $ml^{-1}$, a dose that as shown in Table 1 was able to induce nearly 50% relaxation at both resting tensions. The effectiveness of PAR-2 AP was then tested in the presence of 1 μM diclofenac and 100 μM L-NAME. L-NAME preincubation prevented the PAR-2 AP-mediated relaxation when resting tension was set at 4 g, whereas diclofenac was ineffective. On the contrary, diclofenac fully prevented the relaxing effect of PAR-2 AP at 6 g resting tension, whereas L-NAME preincubation was totally ineffective at this high resting tension (Table 1).

Then, experiments were done with trypsin. Also trypsin can relax tracheas precontracted with ACh in a dose-responsive manner in the range 10–1000 U $ml^{-1}$. In order to better characterise trypsin effectiveness, we performed experiments using 100 U $ml^{-1}$ that corresponded to

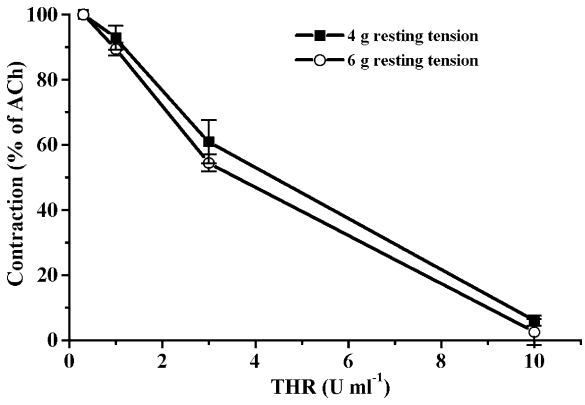


Fig. 4. Effect of thrombin (THR) on 10 nmol $ml^{-1}$ ACh-precontracted guinea-pig tracheal strips. The contraction obtained with ACh was set as 100%. Cumulative dose–response curves of THR were performed at 4 and 6 g resting tension. THR was administered as a bolus. Values are the mean ± s.e.m. of at least four experiments. For more details, see Section 3.

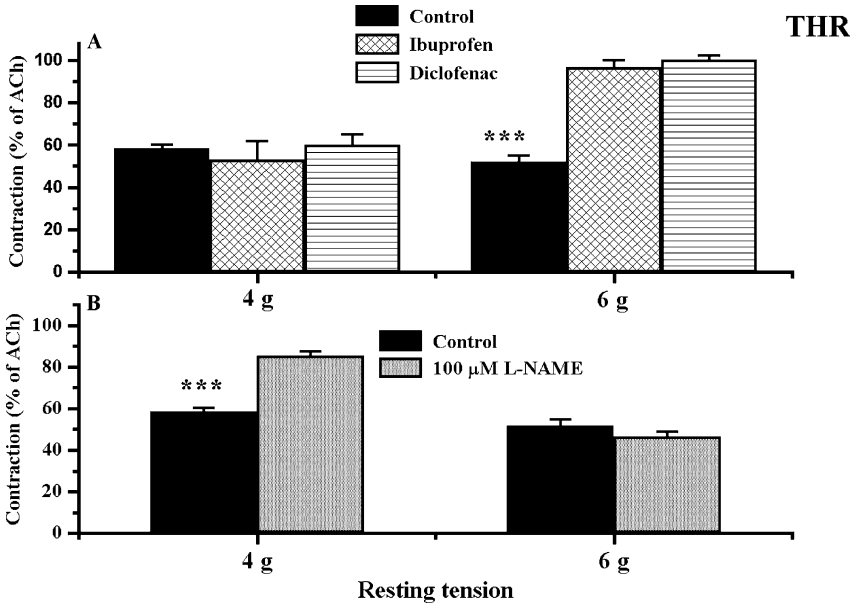


Fig. 5. Effect of COX inhibitors (panel A) and a NOS inhibitor (panel B) on THR-induced relaxation of 10 nmol ml$^{-1}$ ACh-precontracted guinea-pig tracheal strips stretched at 4 and 6 g as indicated. THR (3 U ml$^{-1}$) was administered in control conditions or after 30 min preincubation with 100 μM ibuprofen or 1 μM diclofenac (panel A) or 100 μM L-NAME (panel B). The contraction obtained with ACh was set as 100%. Values are the mean ± s.e.m. of at least four experiments. For more details, see Section 2. Panel A: ***$P<0.001$ vs. ibuprofen and diclofenac, one-way ANOVA followed by Bonferroni's $t$ test. Panel B: ***$P<0.001$ vs. L-NAME, one-way ANOVA.

a suitable median relaxing dose. Data obtained with 100 U ml$^{-1}$ trypsin are summarised in Table 2. The relaxation induced by 100 U ml$^{-1}$ trypsin was very similar at both resting tensions and was partially prevented by 1 μM diclofenac at both resting tensions. Also 100 μM L-NAME partially prevented trypsin-induced relaxation at both resting tensions.

In order to ascertain the crosstalk among the different relaxant agents, the effectiveness of THR, trypsin and ATP was evaluated on ACh-precontracted tracheas already relaxed by 100 nmol ml$^{-1}$PAR-2 AP. Whereas THR 3 U ml$^{-1}$ induced only a small relaxation (15.8 ± 1.65%) of PAR-2 AP relaxed trachea (see Fig. 4 for comparison), trypsin activity was not reduced (50.5 ± 2.52%, $n=4$, see Table 2 for control), but was somehow strengthened. Moreover, in tracheas already relaxed by PAR-2 AP, 100 nmol ml$^{-1}$ ATP was still effective as relaxing agent when resting tension was set at 4 g (23.3 ± 2.09% relaxation obtained after PAR-2 AP, $n=4$ as compared to 25.5 ± 1.39 in control condition, $n=16$, see also Fig. 2). On the contrary, the effectiveness of ATP after PAR-2 AP was reduced at 6 g resting tension (16.5 ± 1.66% relaxation obtained after PAR-2 AP, $n=4$ as compared to 23.5 ± 1.98% in control condition, $n=14$, see also Fig. 2).

### 3.4. Effect of $PGE_2$ and SNAP

In order to better investigate the role of $PGE_2$ on tracheal tone, dose–response curves of $PGE_2$ were also made at 4 and 6 g resting tension. $PGE_2$ (0.001–10 nmol ml$^{-1}$) administered on non-precontracted tracheal strips induced dose-dependent ($r=-0.981$, $P<0.001$), sustained relaxation when resting tension was set at 6 g, the maximal relaxation obtained using 10 nmol ml$^{-1}$ $PGE_2$ being −11.1 ± 2.97 mg mg$^{-1}$ tissue wet weight (Fig. 6). At 4 g resting tension, the dose-dependent ($r=-0.987$, $P<0.001$) decrease in tone was far less pronounced: indeed the maximal relaxation induced by 10 nmol ml$^{-1}$ $PGE_2$ was only −2.0 ± 0.46 mg mg$^{-1}$ tissue wet weight (Fig. 6). When a supermaximal dose of 30 nmol ml$^{-1}$ $PGE_2$ was administered on ACh-precontracted tracheal strips, it induced relaxation that was also resting-tension dependent, being 91 ± 6% at 6 g and only 30 ± 3.5% at 4 g.

Table 1
Relaxing effect of 100 nmol ml$^{-1}$ PAR-2 AP on acetylcholine-precontracted guinea-pig tracheas

| | Relaxation | |
|---|---|---|
| | 4 g | 6 g |
| PAR-2 AP (Control) | 45.9 ± 2.07 (8) | 40.2 ± 3.23 (8) |
| 1 μM Diclofenac + PAR-2 AP | 43.7 ± 1.97 (4) | 2.3 ± 0.18*$^{\S}$ (4) |
| 100 μM L-NAME + PAR-2 AP | 26.0 ± 2.56* (4) | 40.8 ± 3.71 (4) |

The contraction induced by 10 nmol ml$^{-1}$ ACh was set as 100% of contraction. Values are the mean ± s.e.m. of the number of experiments in brackets. *$P<0.05$ vs. the other groups at the same resting tension, one-way ANOVA followed by Bonferroni's $t$ test; $^{\S}P<0.05$ vs. all other groups, one-way ANOVA followed by Bonferroni's test. At both resting tensions, controls performed before either diclofenac or L-NAME did not differ by more than 10% and were therefore cumulated. PAR-2 AP was administered as a bolus on the same ACh-precontracted tracheal strips in control conditions and after preincubation (30 min, perfusion) with either diclofenac or L-NAME as indicated.

Table 2
Relaxing effect of 100 U ml$^{-1}$ trypsin on acetylcholine-precontracted guinea-pig tracheas

| | Relaxation | |
|---|---|---|
| | 4 g | 6 g |
| Trypsin (Control) | 40.0±1.63 (8) | 42.4±1.82 (8) |
| 1 μM Diclofenac+trypsin | 28.8±1.95* (4) | 18.8±2.10* (4) |
| 100 μM L-NAME+trypsin | 24.5±2.23* (4) | 30.5±2.86* (4) |

The contraction induced by 10 nmol ml$^{-1}$ ACh was set as 100% of contraction. Values are the mean±s.e.m. of the number of experiments in brackets. *$P<0.05$ vs. control group at the same resting tension, one-way ANOVA followed by Bonferroni's $t$ test. At both resting tensions, controls performed before diclofenac or L-NAME did not differ by more than 10% and were therefore cumulated. Trypsin was administered as a bolus on the same ACh-precontracted tracheal strips in control conditions and after preincubation (30 min, perfusion) with either diclofenac or L-NAME as indicated.

In a separate set of experiments we tested the effect of NO, using the NO-donor SNAP. Independently of resting tension, SNAP induced a small relaxation on both uncontracted and ACh-precontracted tracheas, its quantifiable effect being only at very high SNAP concentration. Indeed, 100 nmol ml$^{-1}$ SNAP induced a 10% relaxation in ACh-precontracted strips. Also the effect of sodium nitroprusside was scarce and observed only at very high dosage.

### 3.5. $PGE_2$ release from tracheas

As shown in Fig. 7(A), tracheas released a quantifiable amount of $PGE_2$ at both resting tensions in basal conditions. ACh administration (10 nmol ml$^{-1}$) induced a small increase in $PGE_2$ release at both resting tensions

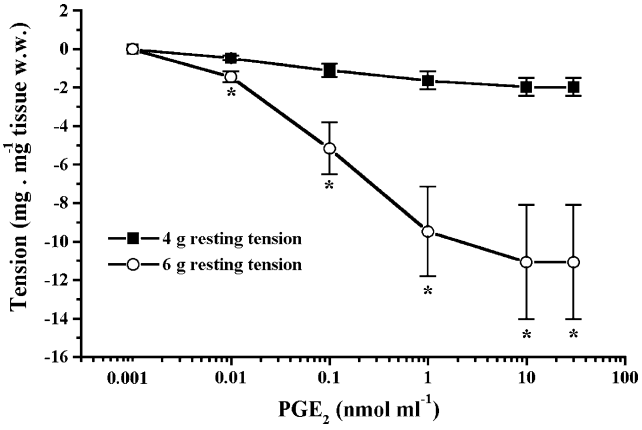


Fig. 6. Effect of prostaglandin $E_2$ ($PGE_2$) on isolated guinea-pig tracheal strips. Cumulative dose–response curves of $PGE_2$ were performed at 4 and 6 g resting tension. $PGE_2$ was administered as a bolus. Values are the mean±s.e.m. of at least four experiments. Basal tension after equilibration was assumed as 0 mg mg$^{-1}$ tissue w.w. Curves were statistically analysed using one-way ANOVA. Curves were significantly different from each other.

(not significant). Administration of ATP to ACh-precontracted strips significantly increased the amount of $PGE_2$ at both resting tensions ($P<0.05$ one-way ANOVA, followed by Bonferroni's $t$ test, Fig. 7(A)), the $PGE_2$ release measured at 6 g being significantly higher than that at 4 g ($P<0.05$ one-way ANOVA, Fig. 7(A)). On the contrary, THR was unable to increase $PGE_2$ at both resting tensions. In the presence of 1 μM diclofenac, $PGE_2$ release was strongly reduced and neither ATP nor THR administered to ACh-precontracted strips were able to significantly increase $PGE_2$ release over basal values (also measured in diclofenac preincubated strips, not shown).

### 3.6. NO release from tracheas

Tracheas stretched at both resting tensions released a measurable quantity of NO in basal conditions (Fig. 7(B)). After ACh administration, the NO release from tracheas was not modified. The administration of either ATP or THR did not change the NO production at both resting tension, but a small, not significant increase in NO release was measured when THR was administered to ACh-precontracted tracheal strips at 4 g but not at 6 g. In the presence of 100 μM L-NAME, the increased quantity of THR was reduced in all conditions (data not shown).

## 4. Discussion

According to our data, in isolated guinea-pig tracheas the relaxation induced by PAR agonists is dependent on epithelial-produced autacoids (i.e. NO and $PGE_2$), but their relative potency as relaxant agonists is dependent on resting tension and therefore on smooth muscle length. This resting tension dependency is a peculiar characteristic of PAR-induced relaxation, while ATP effect is not influenced by it. We would like to point out that the resting tensions used for our investigation were selected to produce a similar contractile effect in the full ACh dose–response range. Moreover, precontracted tracheas with the maximal effective ACh dose can still strongly react to high potassium. Interestingly, according to our preliminary data, at low resting tension (2 g) ACh contraction reaches higher values in the upper part of the dose response curve (4.9±0.25 and 5.1±0.21 at 100 and 300 nmol ml$^{-1}$ ACh, respectively), but both ATP and THR are ineffective in inducing a quantifiable relaxation.

According to the literature, ATP can moderately relax ACh-precontracted preparations in a dose-dependent way [11]. The ATP-induced relaxation is prevented when COX-inhibitors are used. These data strongly indicate that ATP relaxation is fully dependent on the relaxing COX metabolite $PGE_2$. Since ATP is ineffective in epithelial rubbed preparations, $PGE_2$ are probably released from the epithelial layer. These data are in line with previous reports in which ATP can relax guinea-pig tracheal preparations

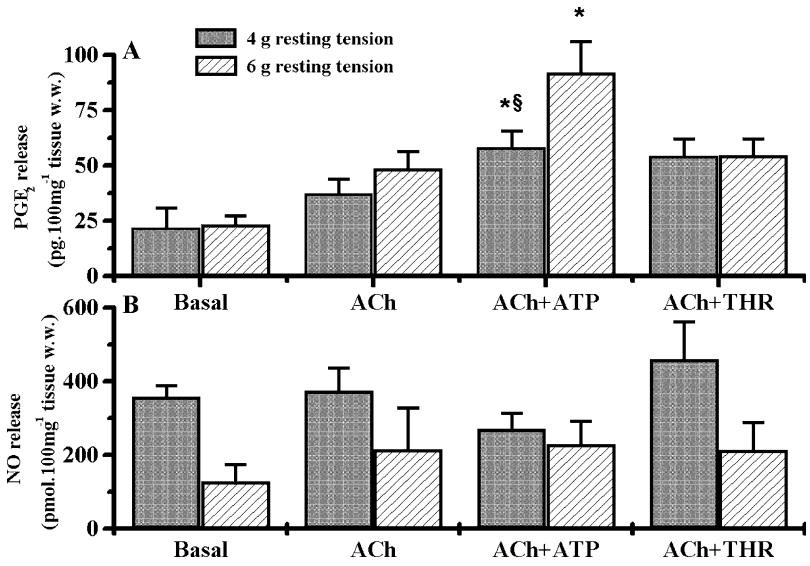


Fig. 7. Release of $PGE_2$ and NO from isolated guinea-pig tracheal strips. Panel A: $PGE_2$ was measured by RIA (performed in duplicate) on the supernatant obtained from isolated guinea-pig tracheal strips preloaded either at 4 or 6 g resting tension and under basal conditions, after administration of 10 nmol $ml^{-1}$ ACh alone (6 min) or after administration of 10 nmol $ml^{-1}$ ACh (3 min) plus 100 nmol $ml^{-1}$ ATP (3 min) or 10 nmol $ml^{-1}$ ACh (3 min) plus 3 U $ml^{-1}$ THR (3 min) as indicated. Basal value indicates the $PGE_2$ amount in the supernatant collected from the closed organ bath (6 min) after tissue equilibration in each experimental condition. For more details, see Section 2. Values are the mean ± s.e.m. of eight experiments. $*P < 0.05$ vs. basal conditions and § vs. ATP 6 g resting tension, repeated measured ANOVA, followed by Bonferroni's *t* test. Panel B: NO was measured by a chemilluminescent method on the supernatant obtained from isolated guinea-pig tracheal strips preloaded either at 4 or 6 g resting tension and under basal conditions, after administration of 10 mmol $ml^{-1}$ ACh alone (6 min) or after administration of 10 mmol $ml^{-1}$ ACh (3 min) plus mmol $ml^{-1}$ ATP (3 min) or 10 mmol $ml^{-1}$ ACh (3 min) plus 3 U $ml^{-1}$ THR (3 min) as indicated. Basal value indicates the NO amount in the supernatant collected from the closed organ bath (6 min) after tissue equilibration in each experimental condition. For more details, see Section 2. Values are the mean ± s.e.m. of six experiments.

precontracted with histamine [10] and ACh [11] in a COX-dependent manner. Moreover, in agreement with the functional experiments the direct quantification of $PGE_2$ production demonstrates that ATP is able to increase $PGE_2$ release at both resting tensions tested. To note that at 6 g, ATP-induced $PGE_2$ release is higher than at 4 g resting tension, but this high production does not increase relaxation. This is also interesting since according to our data exogenous administered $PGE_2$ is more effective at high resting tension.

On the other hand, inhibition of NO production by the NOS inhibitor L-NAME or NO trapping by PTIO do not modify the trachea's sensitivity to ATP relaxation. Moreover, ATP does not modify NO release as determined by the direct dosage. Although the direct dosage NO can suffer from several intrinsic problems, altogether these data indicate that NO release is not involved in ATP-induced relaxation. Therefore, we can conclude that ATP-induced relaxation is fully dependent on $PGE_2$ pathway independently of resting tension.

More intriguing is the relaxant effect of THR, trypsin and PARs agonist peptides. Thrombin is a multifunctional protease that plays important roles in thrombotic, inflammatory, proliferative, and atherosclerotic processes.

The significance of the coagulation factors in the airways is still only partially understood. However, the finding that an increased THR concentration has been measured in the sputum of patients with bronchial asthma [14], in the bronchoalveolar lavage (BAL) of patients with pulmonary fibrosis [15] and allergic asthma after segmental bronchoprovocation (SBP) with antigen [16], suggests that THR may play a role in several inflammatory lung diseases.

According to our data, THR relaxes ACh-precontracted tracheas, its dose–response curves being very similar at both resting tensions. However, at 4 g the THR relaxing effect is not inhibited by COX-inhibitors, whereas the NOS inhibitor L-NAME prevents this relaxation. The involvement of NO in this relaxation is further confirmed by the efficacy of the NO trapping agent PTIO. Conversely, NOS inhibition is ineffective at 6 g, whereas the COX inhibitors totally prevent THR-induced relaxation. These data imply that THR-induced relaxation is dependent on NO pathway at low resting tension relaxation, whereas at higher resting tension, the COX pathway is mainly involved.

Our quantitative results only partially substantiate functional data obtained using THR. Indeed, according to the quantitative dosages, THR determines only a small, not significant increase in NO release at 4 g resting tension and is also unable to significantly increase $PGE_2$ release at 6 g. Although we cannot completely exclude a role of other COX-dependent metabolites in the relaxation induced by THR at 6 g, relaxing lipid autacoids such as prostaglandin $I_2$ are less produced than $PGE_2$ in guinea-pig trachea [17].

However, the direct dosage of autacoid release in the perfusion buffer can only partially reflect the real autacoid production and their concentration in the cellular and

pericellular microenvironment where autacoids can be trapped by several tissue components such as lipids and proteins. In particular NO can rapidly react to thiol groups, producing *S*-nitroso compounds and/or to $O_2$ bubbled in the organ bath as an oxycarb mixture. The high NO reactivity to cellular components and the experimental apparatus could also explain the high variability of the NO dosage.

Our data also show that the relaxant effect of exogenous $PGE_2$ is strongly influenced by resting tension, their effectiveness as relaxing agent in ACh-precontracted preparations being more than 90% when resting tension is set at 6 g, but only 30% when it is 4 g. Therefore, the relevance of the COX pathway in the relaxation induced by THR at 6 g can depend on the great effectiveness of $PGE_2$ at this high resting tension more than a direct increase in its production.

The high relaxant response to exogenously administered $PGE_2$ at high resting tension is quite intriguing. In the guinea-pig trachea, $PGE_2$ administration, by stimulating its EP2 receptor, can increase adenosine $3'{:}5'$ cyclic monophosphate (cAMP, [18]), that phosphorylates several substrates through the activation of protein kinase A (cAK). Among target proteins for cAK, a prominent role is played by calcium-activated potassium channel, the high conductance $Ca^{2+}$-activated $K^+$ ($BK_{Ca}$) channels [19]. Potassium channels can be influenced in a complex way by stretch in smooth muscle cells [20,21] and phosphorylation via different kinases [22,23]. Also, the blockade of $BK_{Ca}$ in guinea-pig trachea can reduce cAMP-dependent relaxation of isolated trachea [24], so it seems likely that the stretch-dependent effect of agonists may be influenced by these mechanisms. Therefore, the different effectiveness of $PGE_2$ according to resting tension may be mediated by the different status of $BK_{Ca}$. Therefore, according to resting tension, $BK_{Ca}$ status can also influence the THR-mediated relaxation. Further functional experiments using specific potassium channel blockers might explore this topic.

NO-releasing drugs such SNAP and sodium nitroprusside have low effectiveness and their action is not influenced by resting tension. However, NO-releasing molecules cannot fully mimic endogenous NO, since kinetic release and cellular localization can differ remarkably.

In our experiments, only the PAR-2 AP relaxes the ACh-precontracted trachea and its action reproduces more the relaxing effect of THR than that of trypsin in regard to the sensitivity of NOS and COX inhibitors, NOS and COX inhibitors preventing the tissue relaxation induced by PAR-2 AP at 4 and 6 g, respectively. Also the results obtained using ATP in tracheas already relaxed by PAR-2 AP are in line with a predominant role of NOS at 4 g and COX at 6 g. Indeed, ATP relaxation (totally dependent on COX at both resting tensions) is unmodified at 4 g, but strongly reduced at 6 g when measured in presence of COX inhibitors.

According to the current literature, THR is a powerful agonist of PAR-1, a weak agonist of PAR-3 and PAR-4, whereas it seems to be ineffective on PAR-2, that can be activated by trypsin and tryptase [25]. However, according to our data, only PAR-2 AP can induce reliable relaxation, whereas the other three peptides are ineffective at concentrations up to 100 nmol $ml^{-1}$. Moreover, our data demonstrate that THR is no far effective as relaxant agent in tracheas already relaxed by PAR-2 AP, thus suggesting a partial or total cross-reactivity on the same receptor subtype. Since PARs antagonists are not well described, we cannot fully address this question. Nevertheless, other authors find a partial cross-reactivity between THR and PAR2 AP [9,26,27]. The PARs-activating peptides used in our experiments can increase intracellular calcium concentration in coronary endothelial cells isolated from the guinea-pig heart according to our standard protocol [28], thus ruling out the possibility that these peptides may be biologically ineffective on guinea-pigs, their potency being as follows: PAR-1 AP $\ggg$ PAR-2 AP $\gg$ PAR-3 AP = PAR-4 AP.

Trypsin can also relax ACh-precontracted strips, its relaxant effect at 100 U $ml^{-1}$ being partially inhibited by diclofenac and L-NAME at 4 and 6 g resting tension. Diclofenac prevents trypsin-induced relaxation more effectively at 6 g, whereas L-NAME is more effective at 4 g. Therefore, the relaxing effect of 100 U $ml^{-1}$ trypsin is dependent on NO production and COX metabolites at both resting tensions. The relaxing effect of trypsin differs from that of THR and PAR-2 AP, suggesting a more complex relationship among receptor subtype activation and intracellular mediators. The finding that trypsin is still fully effective as relaxant agent in tracheas already relaxed by PAR-2 AP, corroborates the hypothesis that trypsin and PAR-2 AP, at least in our experimental conditions, can activate different receptor subtypes. These results suggest that in our model THR can directly activate PAR-2, while trypsin relaxation seems to be partially or totally dependent on other relaxing mechanisms.

In conclusion, our data can explain several discrepancies as regards the responsiveness to PARs agonists in different preparations, where a crucial role is played by resting tension. These results also suggest that endogenous autacoids as well as therapeutic drugs may produce different effects, depending on stretch. These considerations should be taken into account, not only in terms of isolated experiments in vitro, but also in vivo, since cell length in bronchial trees is submitted to natural cyclic changes during breathing. A stretch-dependent dual regulation of the relaxant efficacy of endogenous autacoids (i.e. NO and $PGE_2$) according to smooth muscle length could be a naturally occurring mechanism preventing desensitization of important smooth muscle tone regulators.

Our data can also explain several discrepancies in the literature, since many experimental settings can influence tissue tension and cell length. Indeed, the resting tension and use of contractile agonists can vary the strength that is imposed to tissue preparations.

Moreover, our results may also contribute to clarify the role of proteases in lung pathophysiology. Although all

THR concentrations used in our experiments are lower [16] or higher [15] than those founded in BAL patients with different inflammatory lung diseases, at least in the presence of an intact epithelium, thrombin is able to relax tracheal smooth muscle, thus suggesting a protective role for this protease.

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